

# $\Delta$ Np63 $\alpha$ Is an Oncogene that Targets Chromatin Remodeler Lsh to Drive Skin Stem Cell Proliferation and Tumorigenesis

William M. Keyes,<sup>1,5,\*</sup> Matteo Pecoraro,<sup>2</sup> Victoria Aranda,<sup>1</sup> Emma Vernersson-Lindahl,<sup>1</sup> Wangzhi Li,<sup>1</sup> Hannes Vogel,<sup>3</sup> Xuecui Guo,<sup>1</sup> Elvin L. Garcia,<sup>1</sup> Tatyana V. Michurina,<sup>1</sup> Grigori Enikolopov,<sup>1</sup> Senthil K. Muthuswamy,<sup>1,4</sup> and Alea A. Mills<sup>1,\*</sup>

<sup>1</sup>Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 11724, USA

<sup>2</sup>Centre for Genomic Regulation (CRG), UPF, Barcelona, 08003, Spain

<sup>3</sup>Department of Pathology, Stanford University, Stanford, CA 94305, USA

<sup>4</sup>Ontario Cancer Institute, University of Toronto, Toronto, ON M5G 2M9, Canada

<sup>5</sup>Present address: Centre for Genomic Regulation (CRG), UPF, Barcelona, 08003, Spain

\*Correspondence: [bill.keyes@crg.es](mailto:bill.keyes@crg.es) (W.M.K.), [mills@csHL.edu](mailto:mills@csHL.edu) (A.A.M.)

DOI 10.1016/j.stem.2010.12.009

## SUMMARY

The p53 homolog p63 is essential for development, yet its role in cancer is not clear. We discovered that p63 deficiency evokes the tumor-suppressive mechanism of cellular senescence, causing a striking absence of stratified epithelia such as the skin. Here we identify the predominant p63 isoform,  $\Delta$ Np63 $\alpha$ , as a protein that bypasses oncogene-induced senescence to drive tumorigenesis in vivo. Interestingly, bypass of senescence promotes stem-like proliferation and maintains survival of the keratin 15-positive stem cell population. Furthermore, we identify the chromatin-remodeling protein Lsh as a new target of  $\Delta$ Np63 $\alpha$  that is an essential mediator of senescence bypass. These findings indicate that  $\Delta$ Np63 $\alpha$  is an oncogene that cooperates with Ras to promote tumor-initiating stem-like proliferation and suggest that Lsh-mediated chromatin-remodeling events are critical to this process.

## INTRODUCTION

Deregulation of intrinsic tumor-protective mechanisms can convert normal epithelia into malignant carcinoma and lies at the heart of the majority of human cancers. p53 is a pivotal regulator that protects from such events. Therefore, the discovery of the p53 family members p63 and p73 generated much interest that these proteins would also act to prevent cancer.

However, with regards to p63, a more complicated picture has emerged. p63 is expressed robustly in stem and progenitor cells within the proliferating basal layer of stratified epithelia such as the skin, breast, and prostate. p63's critical importance in epithelia is highlighted by p63-deficient mouse models. In contrast to the viable and fertile, yet tumor-prone, phenotype of p53-deficient mice (Donehower et al., 1992; Jacks et al., 1994), p63-deficient mice die postnatally from severe developmental anomalies, including a lack of skin and associated derivatives, an absence of glands such as prostate and breast, and truncation of limbs (Mills et al., 1999; Yang et al., 1999). We previ-

ously circumvented this postnatal lethality by generating a p63 conditional mouse model (Mills et al., 2002) and reported that inducible ablation of p63 in primary keratinocytes in culture induces a program of cellular senescence and that ablation of p63 in proliferating cells of the adult epidermis in vivo induces cellular senescence and accelerated aging, establishing that p63 deficiency-mediated cellular senescence is a cause of organismal aging (Keyes et al., 2005).

Despite these advances, the role of p63 in cancer has remained perplexing, in large part because multiple p63 isoforms exist. p63 proteins can be classified into two main groups, each of which is transcribed from distinct promoters: those possessing a full-length transactivation domain analogous to that of p53 (referred to as the TA isoform class) and those lacking this domain (referred to as the  $\Delta$ N isoform class) (Yang et al., 1998). Alternative splicing at the carboxy terminus generates three splice variants ( $\alpha$ ,  $\beta$ ,  $\gamma$ ) within each group. The tumorigenic potential of p63-deficient mice has surprisingly not helped to clarify the role of p63 in cancer, because different groups have reported disparate results (Flores et al., 2005; Keyes et al., 2006). In addition, the p63 mouse models used for tumor studies thus far are deficient for all p63 isoforms, and therefore the contribution of individual p63 proteins in cancer may have been masked in these studies. Indeed, it has been suggested that the TA isoforms are most similar to p53, whereas the  $\Delta$ N isoforms have opposing functions.  $\Delta$ Np63 $\alpha$  is the predominant isoform expressed in stem cells as well as in proliferating basal cells of stratified epithelia (Yang et al., 1998).  $\Delta$ Np63 $\alpha$  is overexpressed in some types of human cancer, particularly squamous cell carcinoma (SCC) (Hibi et al., 2000; Tonon et al., 2005), and it has been suggested to have oncogenic functions through inhibition of p53 (Yang et al., 1998). However, in other tumor types, such as adenocarcinoma of the breast and prostate,  $\Delta$ Np63 $\alpha$  expression is lost during the tumorigenic process (Di Como et al., 2002).

Thus, the role of p63 in cancer, and in particular the role of individual p63 isoforms, merits further investigation. Given our previous findings that p63 deficiency induces cellular senescence, we hypothesized that p63 might also be a key modulator of the tumor-suppressive mechanism of oncogene-induced senescence (OIS). We explored the role of p63 in OIS—specifically with activated Ras being the oncogene—with a system

that circumvents the previously generated p63-deficient mouse models. Here we demonstrate that the predominant p63 isoform,  $\Delta$ Np63 $\alpha$ , is an oncogene that promotes stem-like proliferation and carcinoma development through induction of the chromatin remodeler Lsh.

## RESULTS

### Downregulation of $\Delta$ Np63 $\alpha$ Is Required for Oncogene-Induced Senescence

To investigate a potential role for p63 in OIS, we induced senescence in primary mouse keratinocytes by retroviral infection with oncogenic H-Ras-V12 (hereafter referred to as Ras) and monitored p63 expression. As shown previously, Ras induced features of senescence, including proliferation arrest, a characteristic morphology, and enhanced senescence-associated  $\beta$ -galactosidase (SA- $\beta$ -gal) activity (Figure 1; Serrano et al., 1997). Interestingly, induction of senescence was concomitant with a significant decrease in expression of endogenous  $\Delta$ Np63 $\alpha$  expression at both the transcript and protein level, whereas expression of TAp63 isoforms was not affected (Figure 1A; Figure S1 available online). This demonstrates that the predominant p63 isoform expressed in keratinocytes,  $\Delta$ Np63 $\alpha$ , is depleted during OIS.

To determine whether loss of  $\Delta$ Np63 $\alpha$  is a requirement for OIS, primary keratinocytes were coinfectd with Ras and expression constructs encoding  $\Delta$ Np63 $\alpha$ , TAp63 $\alpha$ , or mutant  $\Delta$ Np63 $\alpha$  ( $\Delta$ Np63 $\alpha$ <sup>R279H</sup>). The R279H mutation within the DNA binding domain of p63 causes EEC syndrome in humans and corresponds to one of the most prevalent p53 point mutations found in human cancer (Celli et al., 1999). Whereas expression of  $\Delta$ Np63 $\alpha$  was sufficient to inhibit senescence and induce continued proliferation in Ras-expressing primary keratinocytes, neither TAp63 $\alpha$  nor  $\Delta$ Np63 $\alpha$ <sup>R279H</sup> had this ability (Figures 1B–1D). These data demonstrate that downregulation of  $\Delta$ Np63 $\alpha$  is necessary for OIS and that overexpression of this isoform bypasses senescence.

### $\Delta$ Np63 $\alpha$ Cooperates with Oncogenic Ras to Promote Stem-like Proliferation

Keratinocytes expressing Ras and  $\Delta$ Np63 $\alpha$  (hereafter referred to as R $\Delta$ N cells) had a specific cellular phenotype: whereas shortly after infection they failed to senesce and continued to proliferate slowly, approximately 2 weeks after infection, clonal populations of proliferating cells emerged from these cultures. Given that p63 has been proposed as a determinant of epithelial stem cells (Senoo et al., 2007), we asked whether the clonally proliferating R $\Delta$ N cells that had bypassed senescence had characteristics of stem cells. We grew R $\Delta$ N cells in three-dimensional (3D) culture conditions designed to assay for the self-renewal capability and proliferative potential of stem cells (Figure 2A; Lawson et al., 2007). Whereas vector- or Ras-infected cells failed to proliferate in 3D culture, cells infected with  $\Delta$ Np63 $\alpha$  proliferated and formed clusters containing small numbers of cells. In contrast, the clonal R $\Delta$ N cells that had bypassed senescence proliferated robustly in 3D culture, forming large multicellular spheres. To test whether this sphere-forming capability was a universal feature of cells that had escaped senescence, we inhibited OIS by shRNA-mediated knockdown of p53 in the presence of Ras (referred

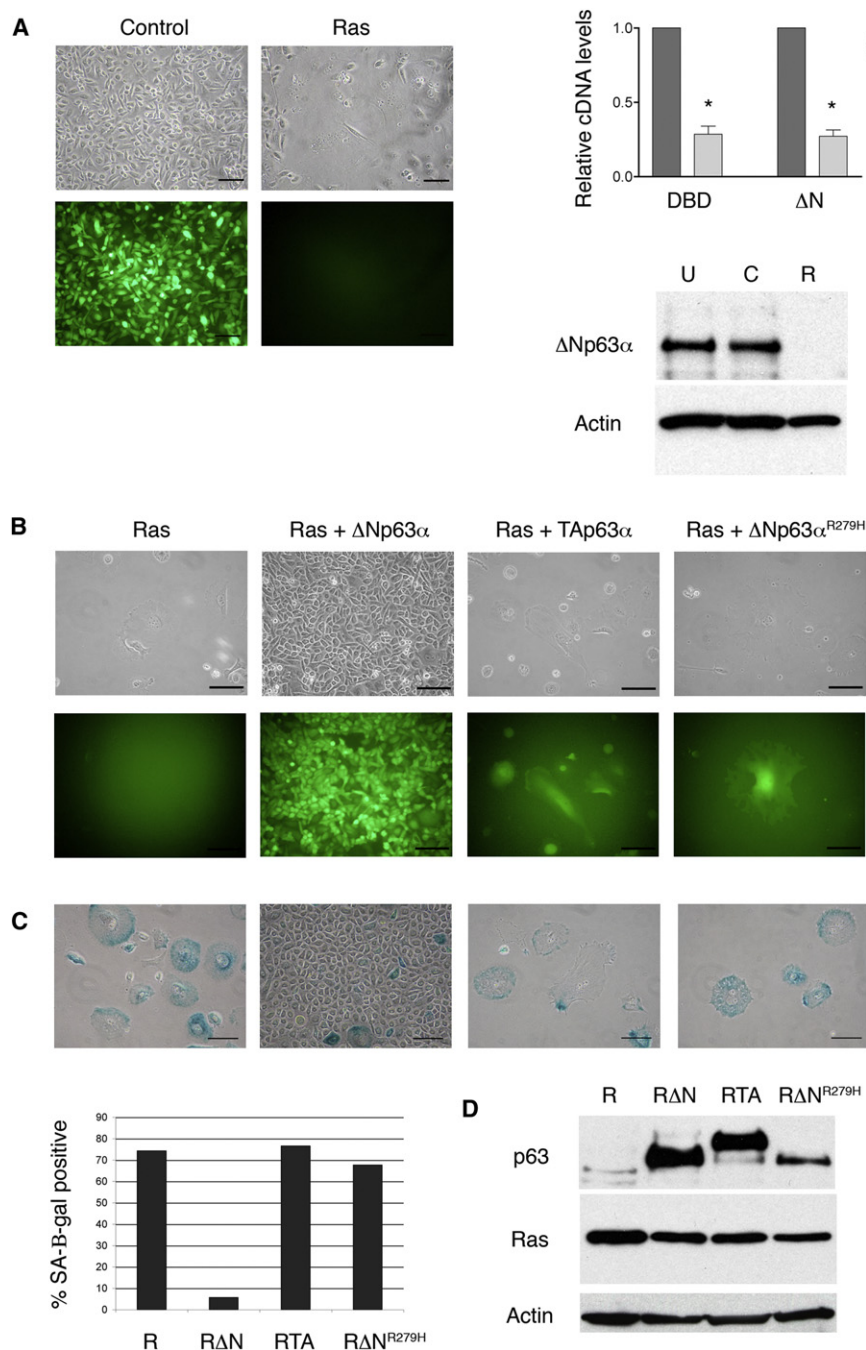
to as Rshp53 cells) and analyzed for growth in 3D culture. Rshp53 cells proliferated robustly and formed large irregular growths, but were devoid of the sphere-forming ability observed in R $\Delta$ N cultures (Figure 2A). This suggested that the 3D sphere phenotype of R $\Delta$ N keratinocytes resulted from a unique cooperation between  $\Delta$ Np63 $\alpha$  and Ras. Immunofluorescent analysis demonstrated that whereas R $\Delta$ N spheres expressed markers of proliferating keratinocytes such as keratin 14 (K14), indicating that they were relatively undifferentiated, Rshp53 cells expressed significantly lower levels of K14 (Figure 2B).

A hallmark feature of stem cells is their ability to self-renew. To assess the self-renewal potential of R $\Delta$ N 3D cultures, primary spheres were either dissociated and replated directly into the 3D culture (data not shown) or were first expanded as a monolayer and then replated into 3D culture (Figure S2A). In each case, R $\Delta$ N cells retained their sphere-forming capability, whereas Rshp53 cells did not have this property. This demonstrates that  $\Delta$ Np63 $\alpha$ 's ability to bypass senescence endows keratinocytes with intrinsic self-renewal capabilities that are characteristic of stem-like cells.

Genes whose expression is elevated in the keratinocyte stem cell populations of mouse skin have been identified (Blanpain et al., 2004; Mignone et al., 2007; Tumber et al., 2004). Interestingly, real-time RT-PCR indicated that 3D cultures of R $\Delta$ N cells expressed elevated levels of a number of stem cell markers relative to Rshp53 cells, including s100a6, ldb2, Notch, and Tcf3 (Figure S3B). By using FACS analysis to identify the CD34/ $\alpha$ -6 integrin double-positive stem cells, we found that the R $\Delta$ N cultures expressed higher levels of the epithelial stem cell markers CD34 and  $\alpha$ 6-integrin than control cultures (not shown). This demonstrates that  $\Delta$ Np63 $\alpha$  acts in concert with oncogenic Ras to promote proliferation of cells with enhanced expression of stem cell-associated genes.

Another key feature of stem cells is their ability to differentiate. We asked whether R $\Delta$ N cells have this capacity by taking advantage of the ability of primary keratinocytes to terminally differentiate and to withdraw from the cell cycle in the presence of elevated calcium (Dotto, 1999). Whereas wild-type keratinocytes lose their epithelial phenotype, acquire a differentiated morphology, and lose expression of proliferation markers such as PCNA upon calcium addition, cells transformed by Rshp53 fail to undergo such differentiation, proliferate robustly, and readily form multiple foci (Figure 2C). Remarkably, R $\Delta$ N keratinocytes were unique: they were similar to wild-type cells with regard to their ability to acquire phenotypic features of terminal differentiation in response to elevated calcium, yet in contrast to wild-type keratinocytes, they did not withdraw from the cell cycle and continued to proliferate. Consistent with these cellular phenotypes, real-time RT-PCR analyses for a panel of markers of epithelial proliferation and differentiation indicates that R $\Delta$ N cells retain both proliferative and differentiation potential (Figure 2D; Figure S2C). These findings demonstrate that whereas both p53 loss and  $\Delta$ Np63 $\alpha$  overexpression facilitate bypass of the tumor-suppressive mechanism of OIS,  $\Delta$ Np63 $\alpha$ -mediated bypass is unique in that it facilitates proliferation while retaining the capacity to differentiate.

The ability of  $\Delta$ Np63 $\alpha$  to enhance stem-like characteristics in the presence of oncogenic Ras could be due to expansion of the normal epithelial stem cell population or alternatively could



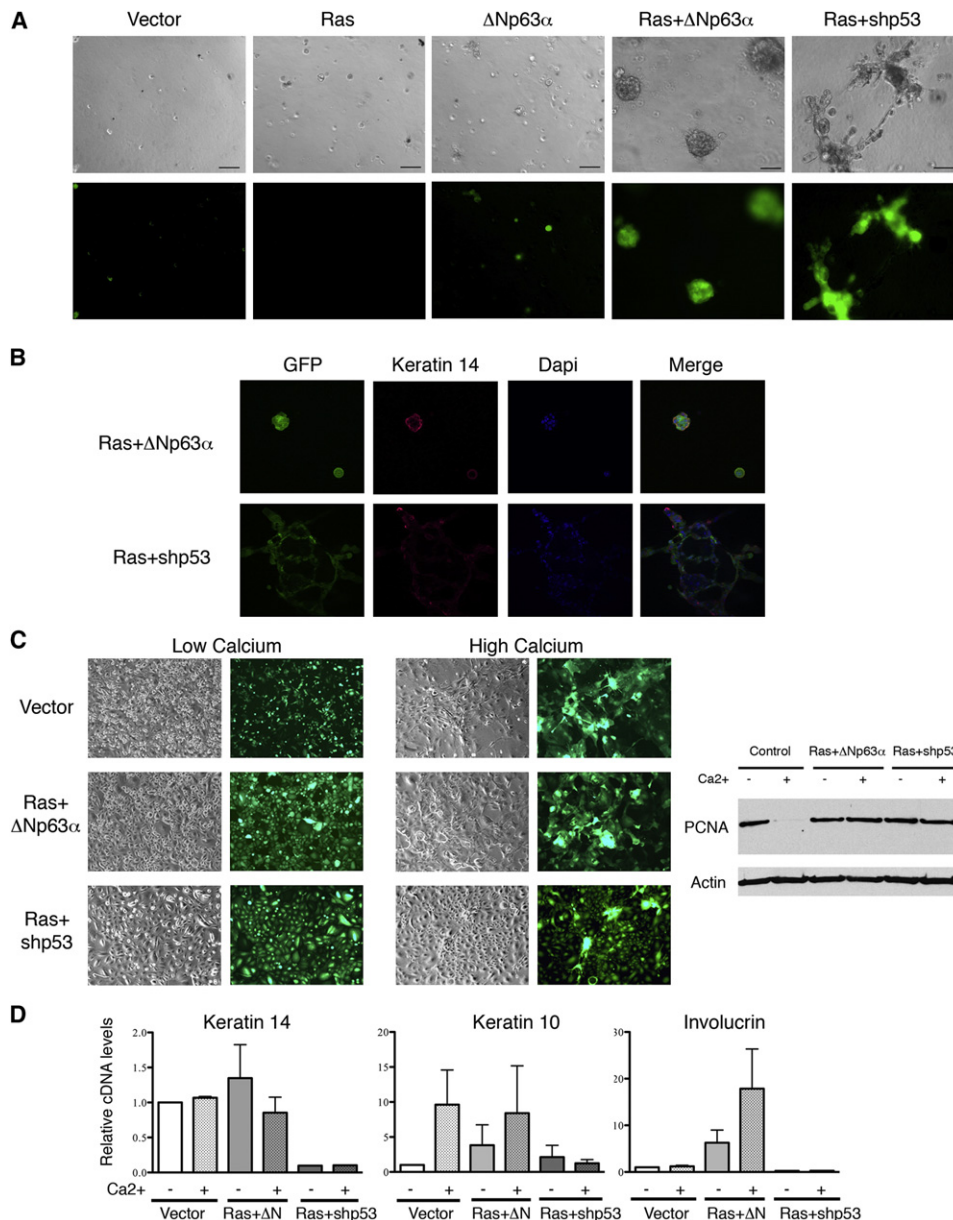
**Figure 1. Downregulation of  $\Delta$ Np63 $\alpha$  Is Required for Oncogene-Induced Senescence**

(A) Primary mouse keratinocytes were infected with retrovirus expressing GFP-vector (control) or oncogenic HRasV12 (Ras), with Ras-infected cells undergoing oncogene-induced senescence (left). qPCR (upper right) and western blot (lower right) analysis 7 days postinfection indicates that  $\Delta$ Np63 is downregulated at both the transcript and the protein level in senescent cells. DNA binding domain, DBD;  $\Delta$ N,  $\Delta$ Np63 isoforms; Uninfected, U; Control, C; Ras, R. Quantitation  $\pm$  SD. Scale bars represent 50  $\mu$ m.

(B) Keratinocytes were infected with retroviral Ras (R) or were coinfecting with Ras as well as GFP-expressing  $\Delta$ Np63 $\alpha$  (R $\Delta$ N), TAp63 $\alpha$  (RTA), or  $\Delta$ Np63 $\alpha$ <sup>R279H</sup> (R $\Delta$ N<sup>R279H</sup>) constructs. Cells expressing Ras and  $\Delta$ Np63 $\alpha$  continue to proliferate. Scale bars represent 50  $\mu$ m.

(C) Senescence-associated- $\beta$ -galactosidase (SA- $\beta$ -Gal) assay and quantitation shows decreased senescence in cells expressing Ras+ $\Delta$ Np63 $\alpha$ . Scale bars represent 50  $\mu$ m.

(D) Western blotting showing expression of p63, Ras, and actin in infected cells.



**Figure 2.  $\Delta Np63\alpha$ 's Ability to Bypass OIS Leads to Proliferation of Cells with Stem-like Properties**

(A) Primary keratinocytes infected with Ras+ $\Delta Np63\alpha$ -GFP form spheres in 3D tissue culture, while cells infected with vector, Ras,  $\Delta Np63\alpha$ , or Ras and a short-hairpin specific for p53 (shp53) do not have this ability.

(B) Immunofluorescence on 3D cultures shows elevated K14 expression in Ras+ $\Delta Np63\alpha$  spheres compared to Ras+shp53 cultures.

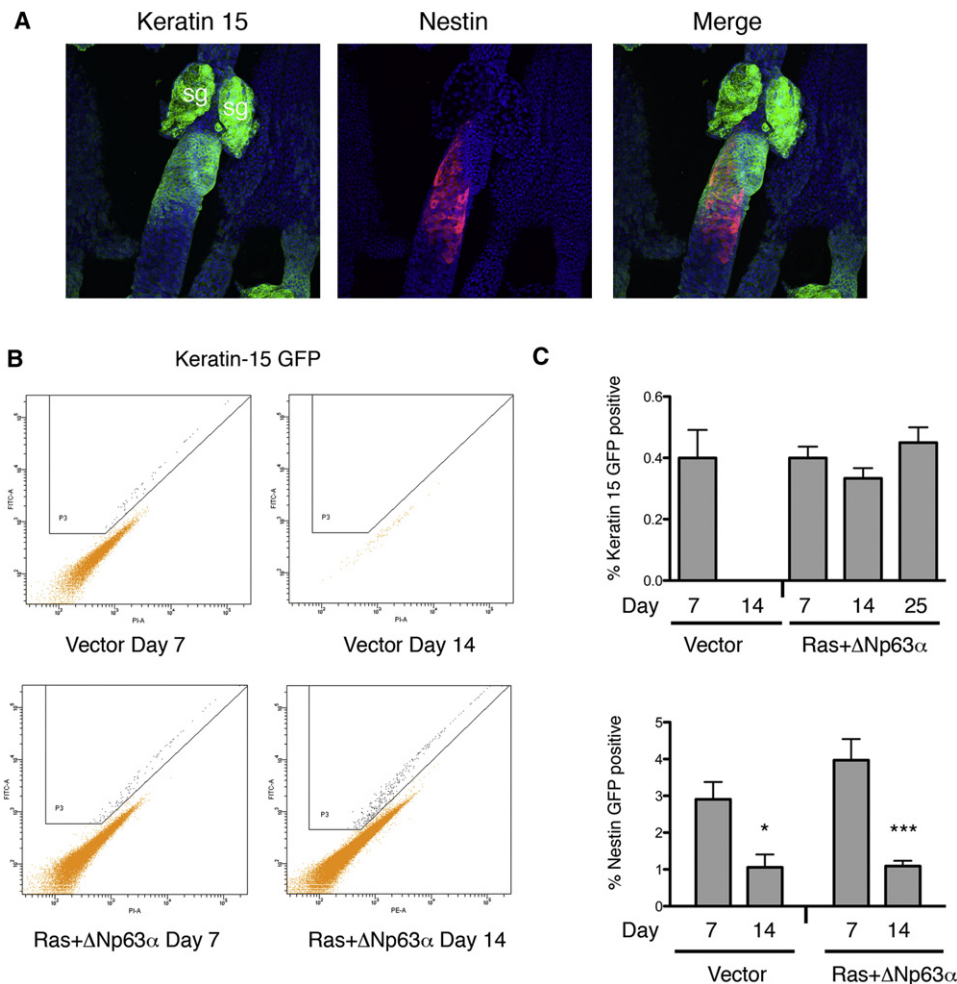
(C) Primary keratinocytes expressing a GFP construct (vector) proliferate in low-calcium media but terminally differentiate and stop proliferating in high-calcium conditions, as shown with western blot for PCNA (right). Cells infected with Ras+ $\Delta Np63\alpha$  acquire the same morphological changes with calcium treatment, but do not stop proliferating, whereas cells expressing Ras+shp53 do not acquire the same differentiation features and remain proliferative.

(D) qPCR for markers of basal epithelia (keratin 14), early differentiation (keratin 10), and late differentiation (involucrin) show that cells expressing Ras+ $\Delta Np63\alpha$  differentiate in response to calcium, similar to early differentiation in vector-expressing cells. Ras+shp53 cells have decreased keratin markers and do not differentiate. Error bars are  $\pm$ SEM.

result from R $\Delta$ N conferring stem-like properties onto the more differentiated transit-amplifying cell population. To determine whether  $\Delta Np63\alpha$  was affecting the normal stem cell population, we used two different mouse models that mark two distinct CD34-positive epithelial stem cell populations of the skin in vivo. The first model is the *keratin 15* (K15)-GFP mouse, in

which the *K15* promoter drives *GFP* expression in stem cells of the hair follicle bulge that can give rise to all epidermal cell types (Morris et al., 2004). The second model is the *Nestin-GFP* mouse, in which the *Nestin* promoter drives *GFP* expression in multipotent CD34-positive, K15-negative stem cells of the hair follicle bulge, which have the capacity to differentiate into multiple





**Figure 3. Ras and  $\Delta$ Np63 $\alpha$  Expression Maintains Survival of K15-Expressing Stem Cells**

(A) Whole-mount immunofluorescence of the bulge region of adult mouse skin identifies keratin15 (green)- and Nestin (red)-positive stem cell populations in the bulge region of the hair follicle. The K15-positive population is visualized with a K15 antibody; the Nestin-positive population is covisualized by immunofluorescence with a GFP-specific antibody in *Nestin-GFP* transgenic mice. Sebaceous glands (sg) label nonspecifically.

(B) FACS analyses of primary keratinocyte cultures derived from *K15-GFP* transgenic mice after infection with vector or Ras+ $\Delta$ Np63 $\alpha$ . After 14 days, cells infected with vector are not maintained and GFP-positive stem cells are depleted. However, cells infected with Ras+ $\Delta$ Np63 $\alpha$  maintain the GFP-positive stem cells 14 days after infection.

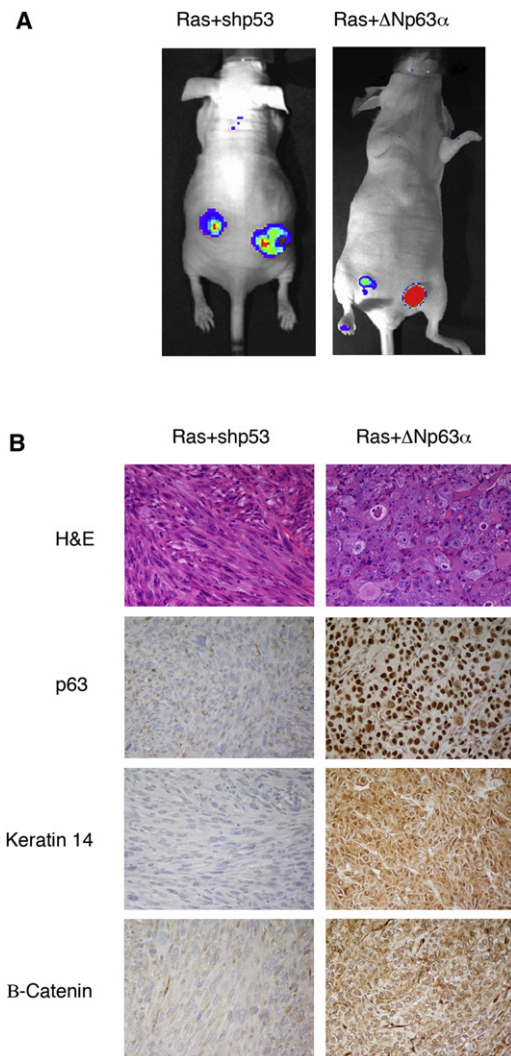
(C) Quantification of FACS experiments (K15,  $n = 5$ ; Nestin,  $n = 9$ ) showing a loss of K15-positive stem cells infected with vector control after 14 days. In contrast, the GFP-positive stem cell population is maintained by Ras+ $\Delta$ Np63 $\alpha$  (top). Similar experiments show Nestin-GFP-positive stem cells (bottom) are not maintained by either vector or Ras+ $\Delta$ Np63 $\alpha$ . Error bars are  $\pm$ SEM.

epidermal cell types, but also have neural, smooth muscle, and adipogenic capacity (Mignone et al., 2007). In both models, GFP-negative cells include committed, more differentiated transit-amplifying cells. Although both K15- and Nestin-positive stem cell populations are prevalent in the hair follicles of the bulge region, these markers identify distinct stem cell populations in adult skin (Figure 3A). We evaluated the effect of  $\Delta$ N on both models by establishing primary keratinocytes from newborn skin from *K15-GFP* and *Nestin-GFP* mice and infecting with control vectors or Ras plus  $\Delta$ Np63 $\alpha$  expression constructs. To evaluate whether the GFP or the non-GFP populations were being affected by  $\Delta$ Np63 $\alpha$ , we assessed the percentage of GFP-expressing cells, specifically looking during both senescence bypass and the clonal expansion stage (Figures 3B and

3C). As expected, the control vector did not confer a proliferative advantage to either the K15- or Nestin-marked stem cell pools, and these cells failed to proliferate 7 days after infection, as is typical for primary mouse keratinocyte cultures. In contrast, the K15-positive (but not the Nestin-positive) stem cell population was maintained in proliferating  $\Delta$ N keratinocytes that had bypassed senescence, even 25 days after infection. These results indicate that  $\Delta$ Np63 $\alpha$  cooperates with oncogenic Ras to maintain the K15-positive stem cell population.

#### **$\Delta$ Np63 $\alpha$ Is an Oncogene that Promotes Tumor Formation**

Our results demonstrate that expression of  $\Delta$ Np63 $\alpha$  inhibits OIS and promotes proliferation of cells with properties of stem cells.



**Figure 4.  $\Delta$ Np63 $\alpha$  Is an Oncogene that Cooperates with Oncogenic Ras In Vivo**

(A) Whole-animal imaging of nude mice injected subcutaneously with primary keratinocytes expressing Ras+shp53-GFP or Ras+ $\Delta$ Np63 $\alpha$ -GFP shows the development of GFP-expressing tumors.

(B) Pathological and immunohistological analyses demonstrate distinct tumor types. Ras+shp53 tumors present mainly as fibrosarcoma lacking expression of epithelial markers, whereas Ras+ $\Delta$ Np63 $\alpha$  tumors are squamous cell carcinomas expressing p63, K14, and  $\beta$ -catenin.

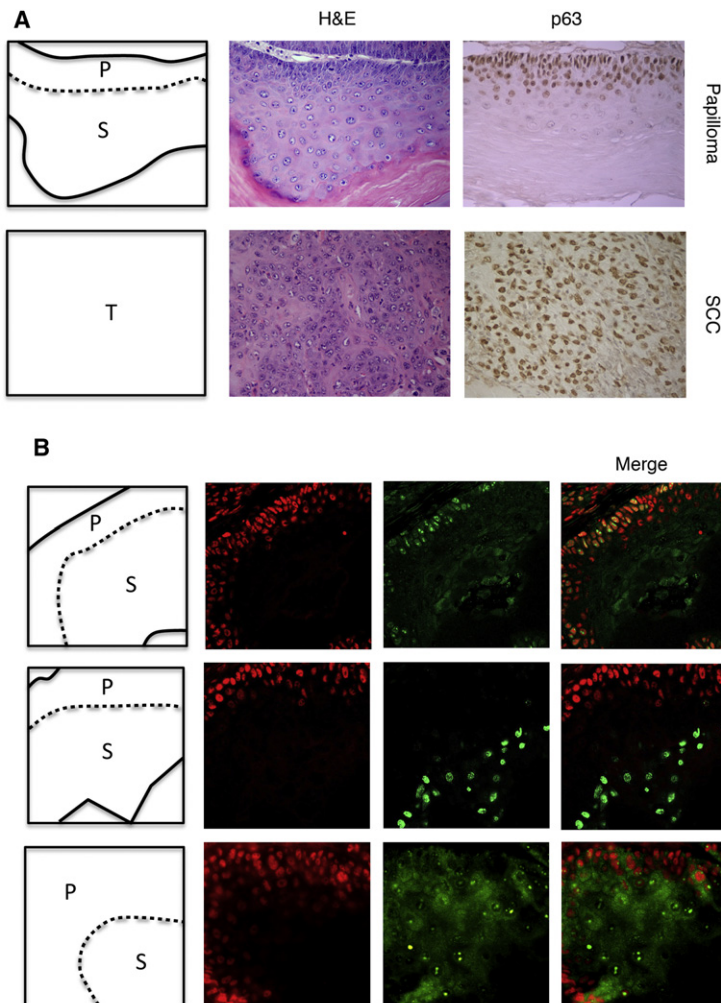
Aberrantly proliferating stem cells, or cells that have acquired stem-like features, have been suggested to be the cell-of-origin for a number of tumor types. Mutations that impair senescence can facilitate malignancy, so we asked whether primary cells infected with R $\Delta$ N were transformed and tumorigenic in vivo. As positive controls, primary keratinocytes infected with Ras and shRNA-p53-GFP were injected subcutaneously into nude mice. Imaging and analysis of these mice revealed the growth of GFP-positive tumors with the expected high incidence for cells transformed by p53 deficiency in the context of Ras (overall tumor incidence, 17/18 sites, 94%) (Figure 4A; Table S1). Injection of cells infected with either vector, Ras alone, or  $\Delta$ Np63 $\alpha$

alone did not yield any tumors, in agreement with previous results (Table S1; Missero et al., 1996). Strikingly, R $\Delta$ N cells also formed GFP-positive tumors, albeit with a lower overall incidence (12/24 sites, 50%) (Figure 4A; Table S1), indicating that  $\Delta$ Np63 $\alpha$  is an oncogene that induces tumorigenesis in cooperation with oncogenic Ras.

Histopathological analyses of tumors that developed in vivo revealed that R $\Delta$ N expression caused tumors that were histologically distinct from those in which p53 was knocked down, even though the same preparations of cells that had been cultured under identical conditions in parallel were used. Tumors that developed from Rshp53 cells were large, rapidly growing hard masses and presented primarily as fibrosarcoma or poorly differentiated sarcoma, with features of having undergone epithelial-mesenchymal transformation (EMT) (Figure 4B; Table S1). In contrast, tumors that developed from R $\Delta$ N cells were slower growing, often included smaller tumor masses surrounded by necrotic tissue, and presented predominantly as SCC, with some mice developing poorly differentiated sarcoma. Immunohistochemical analyses further demonstrated the distinction between the different tumor types, with the R $\Delta$ N tumors expressing the epithelial markers K14 and  $\beta$ -catenin, while these epithelial markers were not detectable in Rshp53 tumors (Figure 4B; Figure S3A). Interestingly, N-cadherin, a marker of EMT, was detectable in only a few cells of R $\Delta$ N tumors, but was expressed robustly throughout Rshp53 tumors (Figure S3A), suggesting that R $\Delta$ N had not undergone EMT. Furthermore, whereas immunostaining for p53 and p19 showed that p53-deficient tumors had elevated p19 expression, indicating that p53-mediated feedback inhibition of p19 was impaired, R $\Delta$ N tumors were also deficient for p53, but p19 was not detectable in these tumors (Figure S3B). This further demonstrates the histological and molecular distinction between these tumors. These findings are in agreement with the high incidence and early expression of  $\Delta$ Np63 $\alpha$  in human SCC (Hibi et al., 2000), demonstrating that  $\Delta$ Np63 $\alpha$  is an oncogene capable of causing tumor development in a manner distinct from deficiency of p53.

#### Downregulation of p63 Occurs in Senescent Cells of Premalignant Lesions In Vivo

To extend our findings that p63 depletion is a prerequisite for OIS, we monitored expression of endogenous p63 in vivo in a model of OIS bypass-induced carcinogenesis. Topical treatment of mouse skin with DMBA induces Ras mutations, which facilitate development of premalignant papillomas with markers and features of OIS; inactivation of mediators of OIS facilitates senescence bypass and converts preneoplastic lesions to malignant SCC (Collado et al., 2005). We examined skin sections from DMBA-induced papillomas and SCC for expression of p63 and markers of senescence. p63 was detectable in premalignant lesions, as previously shown (Keyes et al., 2006), but notably was most robust in the proliferating basal layer, with expression decreasing in suprabasal layers (Figure 5), analogous to the graded pattern of p63 observed in normal skin. This contrasts markedly with p63 expression in SCC, which is abundant in transformed cells that escaped senescence and progressed to malignancy (Figure 5A). To examine whether the decrease in p63 expression in the premalignant lesions correlated with cells that had undergone OIS, tissues were costained for p63 as well



**Figure 5. Downregulation of Endogenous p63 Occurs in Senescent Cells of Premalignant Lesions In Vivo**

(A) Papilloma and SCC were induced by treating mouse skin with DMBA. In papillomas, p63 expression is restricted to the basal layer, but decreases in the lesion, whereas p63 is expressed in all cells that progressed to squamous cell carcinoma (SCC). The schematic highlights areas of proliferation (P), senescence (S), and tumor (T).

(B) Immunohistochemical analyses of premalignant papillomas indicate that Ki67 and p63 are coexpressed in the basal layer but are absent in the nonproliferative region where the senescence markers  $\gamma$ H2AX and p19 are expressed. The schematic highlights areas of proliferation (P) and senescence (S).

not only indicate that the initiating events by which  $\Delta$ Np63 $\alpha$  inhibits senescence probably do not occur via these pathways, but it also suggests that additional as yet uncharacterized senescence pathways are modulated by  $\Delta$ Np63 $\alpha$ .

To identify genes whose expression was altered by  $\Delta$ Np63 $\alpha$  expression and hence might reveal novel  $\Delta$ Np63 $\alpha$ -mediated pathways facilitating senescence bypass, we performed cDNA microarray analyses on primary keratinocytes at an early stage after infection with R $\Delta$ N and controls. This led to the identification of 26 genes whose expression was either increased (23 genes) or decreased (3 genes) in R $\Delta$ N cells relative to controls (Figure S4B). Real-time RT-PCR confirmed that expression of a number of these genes was altered in R $\Delta$ N cells, indicating that these genes are induced during the initial phase of senescence bypass.

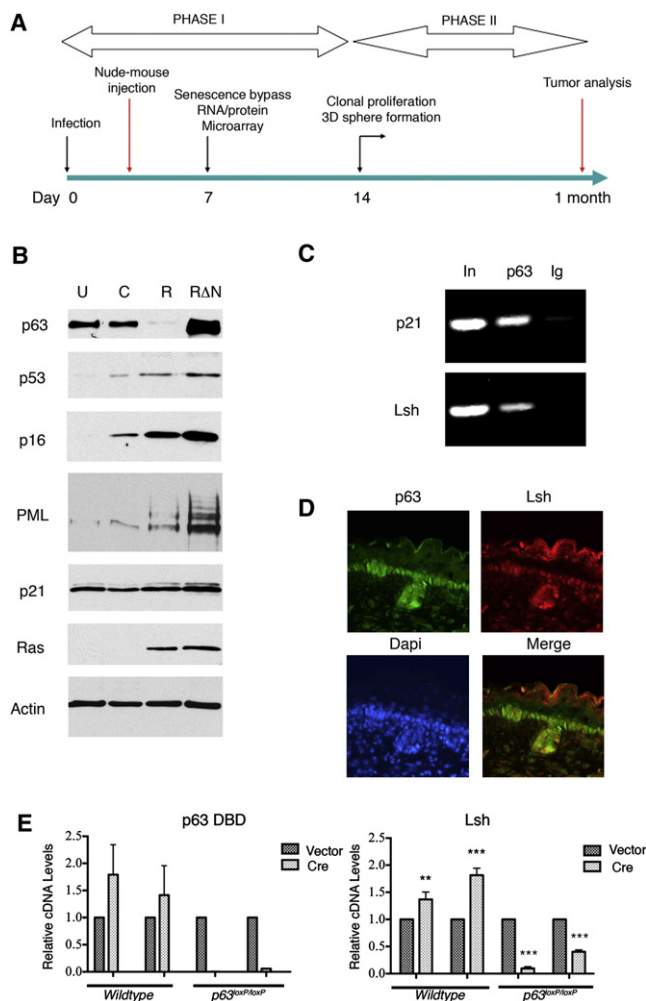
as for markers of proliferation and senescence (Figure 5B). Whereas expression of p63 overlapped with the proliferation marker Ki67, it did not overlap with the senescence markers  $\gamma$ H2AX and p19. This indicates that OIS in vivo occurs in p63-depleted cells, whereas cells that bypass senescence and develop into tumors express p63 robustly. The finding that the patterns of expression of p63 and markers of senescence are mutually exclusive in vivo confirms and extends our findings that downregulation of endogenous  $\Delta$ Np63 $\alpha$  is required for OIS.

#### Lsh Is a p63 Target Gene

To elucidate the mechanism by which  $\Delta$ Np63 $\alpha$  inhibits OIS, we examined primary keratinocytes that had been infected with R $\Delta$ N or control vectors shortly after infection (see schematic, Figure 6A). This time point precedes the development of distinct cellular phenotypes and was chosen because we wanted to identify  $\Delta$ Np63 $\alpha$ -modulated pathways that initiated senescence bypass and led to stem-like proliferation and tumorigenesis. We examined expression of known senescence mediators at the transcript and protein level in R $\Delta$ N cells; however, p53, p16, p19, p21, and PML were not reduced during the initial stages of senescence bypass (Figures 6B; Figure S4A). These analyses

To determine whether any of the candidate genes were direct targets of  $\Delta$ Np63 $\alpha$ , we analyzed the regions upstream of the transcriptional start sites for p53/p63 consensus binding sites. We identified a p63-consensus binding site in the mouse *Lsh* (lymphoid-specific helicase) gene (Figure S5A), which encodes a member of the SNF2 family of chromatin remodeling proteins. Like p63, Lsh has been implicated in both embryonic development and cellular senescence (Geiman et al., 2001). Analysis of the human *LSH* promoter identified putative p63 consensus binding sites similar to those described previously (Figure S5B; Yang et al., 2006). To determine whether p63 binds the *Lsh* promoter, we performed chromatin immunoprecipitation (ChIP) in R $\Delta$ N cells with an antibody specific for p63. Primers for p21, a previously identified  $\Delta$ Np63 $\alpha$  target gene, were used as a positive control (Figure 6C). By using multiple primer pairs flanking the p63 consensus binding site identified in *Lsh*, we found that p63 bound this region, whereas under similar conditions, we did not detect binding to the promoter region of two other genes (Figure 6C; Figure S5C). The p63 antibody used for ChIP was raised against a peptide common to each of the six p63 isoforms (Yang et al., 1998). To determine which p63 isoform was probably directly regulating *Lsh*, we performed western analyses of





**Figure 6. *Lsh* Is a p63 Target Gene**

(A) Timeline schematic of the experimental procedure. Phase I corresponds with initiating events leading to senescence bypass, whereas phase II involves clonal proliferation of stem-like cells and tumor development.

(B) Western blotting for p63 and senescence mediators in cells expressing Ras+ΔNp63α during phase 1 of senescence bypass indicates that expression of p53, p16, PML, or p21 are not reduced.

(C) Chromatin immunoprecipitation (ChIP) analysis shows p63 is bound to the *Lsh* promoter. p21 binding serves as a positive control. In, input; Ig, immunoglobulin (negative) control.

(D) Immunofluorescence for p63 and Lsh in E17.5 mouse skin shows overlap in the basal epithelia.

(E) Primary mouse keratinocytes from two individual wild-type and *p63<sup>loxP/loxP</sup>* mice were infected with retroviral vector or Cre. Expression of p63 DNA-binding domain (DBD) is lost in *p63<sup>loxP/loxP</sup>* cells with Cre, and *Lsh* expression is decreased coordinately. Error bars are ±SEM.

lysates that had been immunoprecipitated with the p63 antibody. This analysis revealed that ΔNp63α is the predominant isoform detectable in these cells (Figure S5D), supporting the idea that *Lsh* is a direct target of this isoform.

To determine whether ΔNp63α and *Lsh* are expressed in the same cell type in an in vivo setting, we assessed the pattern of *Lsh* expression in embryonic day 17.5 skin, a time point when ΔNp63α levels are especially robust (Figure 6D).

Coimmunostaining revealed a clear overlap between p63 and *Lsh* expression in proliferating basal keratinocytes of the epidermis, further supporting the fact that *Lsh* is a target of ΔNp63α. To assess whether ΔNp63α is required for *Lsh* expression, we ablated p63 in primary keratinocytes obtained from the *p63* conditional mouse model that we described previously (Keyes et al., 2005; Mills et al., 2002). Indeed, Cre-mediated disruption caused loss of p63, while simultaneously reducing *Lsh* expression at the transcript level (Figure 6E). These findings demonstrate that the pattern of *Lsh* expression overlaps with that of ΔNp63α in vivo and that *Lsh* expression is dependent upon p63 expression, providing additional evidence for *Lsh* being a direct target of ΔNp63α in primary keratinocytes.

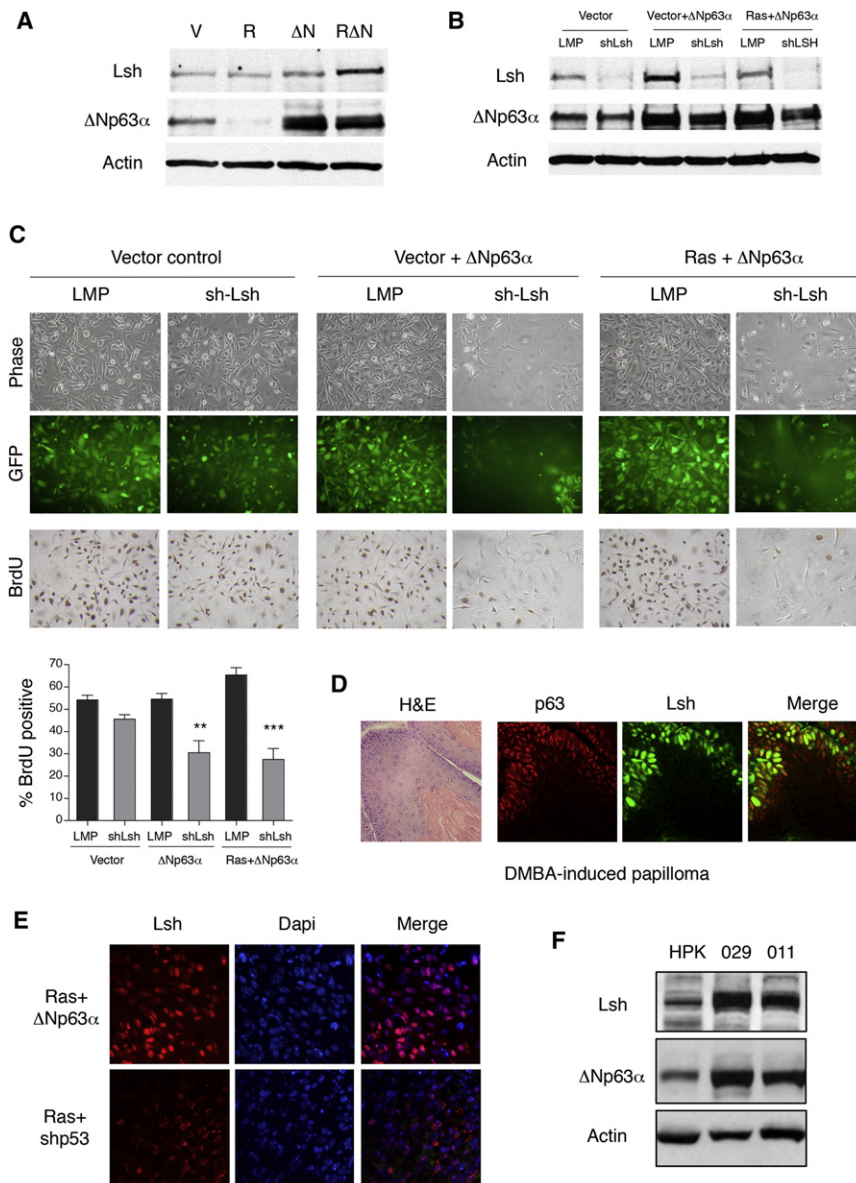
### Lsh Is Required for ΔNp63α-Mediated Senescence Bypass

Having identified *Lsh* levels as being upregulated in RΔN cells at the transcript level, we examined *Lsh* expression at the protein level. We prepared protein lysates from ΔNp63α, RΔN, and control cells 6 days after infection, at a time point prior to senescence bypass when all cultures were phenotypically similar (see Figure 6A). Western analyses indicated that *Lsh* protein levels were higher in cells overexpressing ΔNp63α (Figure 7A). In addition, we found that in contrast to control cultures, RΔN keratinocytes could be maintained for an extended time in culture, and these cells expressed robust levels of *Lsh* (Figure S6A). These findings further support our observations that ΔNp63α mediates *Lsh* expression and that *Lsh* is induced prior to, and is maintained after, senescence bypass.

Our findings identify *Lsh* as a p63 target whose expression is induced during senescence bypass and tumor initiation. To determine whether *Lsh* is required for ΔNp63α-mediated inhibition of OIS, we generated multiple shRNAs for *Lsh*. We identified one hairpin (977) that was capable of reducing *Lsh* levels by about 95% (Figure 7B; Figure S6B). To test the specificity of this hairpin, we assayed for *Lsh* activity by assessing reactivation of repetitive elements throughout the genome—integrated retrotransposons silenced by *Lsh* (Figure S6C; Sun et al., 2004). A number of repeat elements (including *ERV1*, *MaLR*, *major* and *minor satellites*, *LINE1*) were significantly upregulated in *Lsh*-depleted cells, providing functional evidence that *Lsh* activity is compromised by RNAi-mediated knock down.

To functionally test whether *Lsh* was required for ΔNp63α-mediated bypass of senescence, keratinocytes were infected with combinations of vector controls, Ras, ΔNp63α, and shRNA-*Lsh* and the extent of RΔN-mediated bypass of senescence was evaluated (Figures 7B and 7C). Interestingly, whereas knockdown of *Lsh* in vector control cells impaired proliferation (Figure 7C), in agreement with the finding that deficiency of *Lsh* induces senescence in vivo (Sun et al., 2004), knockdown of *Lsh* in the context of ΔNp63α produced a stronger senescent-like morphology, irrespective of Ras expression, a finding consistent with *Lsh* being a target of ΔNp63α. In agreement, a specific hairpin that did not affect *Lsh* levels did not have this effect (not shown). BrdU incorporation shortly after infection indicated that proliferation was significantly compromised when *Lsh* was knocked down in the presence of ΔNp63α, with remaining cells failing to proliferate when maintained for longer periods.





**Figure 7. Lsh Is Required for  $\Delta$ Np63 $\alpha$ 's OIS-Inhibiting Activity**

(A) Western blotting for Lsh and p63 in primary mouse keratinocytes expressing vector (V), Ras (R),  $\Delta$ Np63 $\alpha$  ( $\Delta$ N), and Ras+ $\Delta$ Np63 $\alpha$  (R $\Delta$ N) during phase I senescence bypass indicates that Lsh expression is increased in  $\Delta$ Np63 $\alpha$ -expressing cells.

(B) Western blotting for Lsh and p63 in primary mouse keratinocytes expressing vector (V),  $\Delta$ Np63 $\alpha$  ( $\Delta$ N), and Ras+ $\Delta$ Np63 $\alpha$  (R $\Delta$ N) coinfecting with retroviral vector (LMP) or shRNA-Lsh.

(C) Cell morphology by phase contrast and GFP-fluorescence shows fewer cells and senescent-like morphology in Lsh-knockdown cells. Immunostaining for BrdU incorporation and quantitation shows that Lsh-knockdown cells expressing  $\Delta$ Np63 $\alpha$  proliferate less. BrdU quantitation,  $\pm$  SEM: one-way ANOVA/Tukey's t test.

(D) Immunostaining indicates that p63 and Lsh are coexpressed in DMBA-induced premalignant papillomas in situ.

(E) Immunostaining indicates that Lsh is expressed robustly in tumors arising from subcutaneous injection of R $\Delta$ N keratinocytes, but not those arising from subcutaneous injection of Rshp53 keratinocytes.

(F) Western blotting shows increased Lsh expression in two squamous cell carcinoma cell lines (029, 011) that have increased p63 expression, compared to human primary keratinocytes (HPK).

These data indicate that the  $\Delta$ Np63 $\alpha$  target Lsh is an essential mediator of senescence bypass.

To assess the relationship between endogenous p63 and Lsh in senescence inhibition and tumorigenesis in an in vivo setting, we analyzed the pattern of Lsh expression in DMBA-induced premalignant papillomas. Whereas Lsh was not detectable in normal skin of these adult animals (Figure S7A), it was robustly expressed in the p63-positive proliferative zone of premalignant lesions; in contrast, Lsh was not detectable in the p63-negative senescent zone (Figure 7D). This coexpression of p63 and Lsh in chemically induced carcinomas of the skin was in agreement with our analyses of R $\Delta$ N and Rshp53 tumors obtained in nude mice, as shown by the fact that R $\Delta$ N tumors had significantly higher expression of Lsh (Figure 7E; Figure S7B). To examine whether  $\Delta$ Np63 $\alpha$  and LSH expression are also coregulated in human carcinomas, we analyzed HNSCC cell lines previously

stem-like proliferation, setting the stage for carcinoma development in vivo.

## DISCUSSION

Our study identifies  $\Delta$ Np63 $\alpha$  as an oncogene in the skin, capable of inhibiting oncogene-induced senescence and promoting development of squamous cell carcinoma. There has been much confusion about the role of p63 in tumorigenesis. Unlike p53, point mutations in p63 are rarely associated with cancer, but cause a spectrum of developmental birth defects (Brunner et al., 2002). In further contrast to p53, the predominant p63 isoform detectable in proliferating epithelia,  $\Delta$ Np63 $\alpha$ , is frequently overexpressed in a variety of human tumors, particularly SCC of the skin, head and neck, and lung (Hibi et al., 2000; Tonon et al., 2005), although an oncogenic role for  $\Delta$ Np63 $\alpha$  had not

been demonstrated previously. In the context of cultured cells,  $\Delta$ Np63 $\alpha$  was suggested to promote oncogenesis by inhibiting p53 activity (Yang et al., 1998), though this has not been demonstrated in vivo. However, we found that primary epithelial cells transformed with these different genetic lesions give rise to cells and tumors with different properties, suggesting that  $\Delta$ Np63 $\alpha$  promotes tumor development through p53-independent mechanisms. Our data now conclusively show that gain of  $\Delta$ Np63 $\alpha$  activity is a causative lesion that cooperates with oncogenic Ras to promote tumorigenesis. Furthermore, the ability of  $\Delta$ Np63 $\alpha$  to inhibit OIS provides mechanistic insight that explains why overexpression of this isoform in human tumors is an early and highly penetrant event.

We had previously discovered that the apparent tumor protection in p63-compromised mice comes at the expense of reduced lifespan (Keyes et al., 2005). Furthermore, conditional ablation of p63 in stratified epithelia does not induce tumorigenesis (Mills et al., 2002; W.M.K. and A.A.M., personal observation), but in contrast, activates the tumor-suppressive mechanism of cellular senescence, interestingly, with features of accelerated aging (Keyes et al., 2005). This provided functional evidence that cellular senescence and aging are linked in vivo and exemplified p63's role in this process. All p63 proteins were ablated in these studies, so the contribution of individual p63 isoforms was not established. We hypothesized that loss of  $\Delta$ Np63 $\alpha$  was responsible for p63 deficiency-induced cellular senescence, an idea supported by the finding that  $\Delta$ Np63 $\alpha$  regulates epithelial stem/progenitor cells within the epidermis (Senoo et al., 2007). A recent report suggests that the TAp63 isoforms also function to regulate senescence within a different stem cell niche, inhibiting cellular senescence in skin-derived progenitor cells (SKPs) (Su et al., 2009)—a progenitor population that gives rise to neuronal and mesenchymal cell types, but not to epithelial cell types such as keratinocytes (Fernandes et al., 2008; Toma et al., 2005). In yet another setting—fibroblasts—TAp63 isoforms induce cellular senescence in a p53-independent manner (Guo et al., 2009). These data highlight the roles of p63 in specific stem/progenitor cell populations of distinct tissues, as we now show occurs in tumor-initiating stem-like cells. The findings that distinct p63 isoforms regulate cellular senescence differently within diverse settings suggest that although senescence is a program that has common mediators and pathways, there are underlying tissue-specific mechanisms at play.

A major finding of this study is that it identifies  $\Delta$ Np63 $\alpha$  as an inhibitor of OIS. OIS is a tumor-suppressive mechanism that curtails aberrant proliferation of cells responding to DNA damage or oncogenic signaling, a program mediated predominantly by tumor suppressors such as p53, p16, and Rb (Narita and Lowe, 2005). We had previously linked p63 deficiency to cellular senescence (Keyes et al., 2005) and more recently found that TAp63 isoforms are essential inducers of OIS that halt both the initiation and progression of Ras-driven p53-deficient tumors in vivo (Guo et al., 2009). This ability of TAp63 to induce OIS in mesenchymal cells contrasts vividly with the potent senescence-inhibitory activity of  $\Delta$ Np63 $\alpha$  in epithelial cells presented here, underscoring the unique role of different p63 isoforms in distinct cellular contexts.

We used an unbiased genetic screen to uncover mechanisms by which  $\Delta$ Np63 $\alpha$  bypasses senescence and induces stem-like

proliferation. This approach revealed a number of interesting candidates, including stem cell-associated genes (*Brca1*, *Geminin*, *Lsh*, *TGF- $\beta$* ), DNA damage and repair genes (*Brca1*, *Chk1*, *Exo1*, *Rad51*, *Cdc6*), and others, suggesting that  $\Delta$ Np63 $\alpha$  affects cellular proliferation on several levels. Interestingly, mouse models deficient for three of these candidates have phenotypes of cellular senescence or organismal aging: *Lsh* (Sun et al., 2004), *Exo1* (Schaezlein et al., 2007), and *Brca1* (Cao et al., 2003). Here we identify *Lsh*, a chromatin remodeler of the SNF2 family, as a p63-induced protein required for  $\Delta$ Np63 $\alpha$ -mediated senescence bypass. *Lsh* plays a key role in senescence and aging, with *Lsh*-deficient mice exhibiting either postnatal lethality (Geiman et al., 2001) or aberrant aging and enhanced cellular senescence (Sun et al., 2004). *Lsh* regulates DNA methylation and transcriptional silencing (Muegge, 2005), as indicated by the fact that its deficiency causes DNA hypomethylation, altered histone modification, and alterations in heterochromatin (Dennis et al., 2001; Muegge, 2005). In particular, *Lsh* is required for methylation-induced silencing of repeat elements and transposons in the pericentromeric regions (Huang et al., 2004; Sun et al., 2004) and also causes transcriptional silencing of individual loci including *Cdkn1c/p57* (Sun et al., 2004) and *Hox* (Xi et al., 2007) genes. *Lsh* functions by recruiting DNA methyltransferases (Myant and Stancheva, 2008) or through direct interaction with members of the Polycomb repressive complex 1 (Xi et al., 2007). Although the mechanism by which  $\Delta$ Np63 $\alpha$ -driven *Lsh* expression facilitates senescence bypass and tumor development remain to be elucidated, a recent report that *Lsh* is overexpressed in human HNSCC (Gemenetzi et al., 2009) supports our finding that  $\Delta$ Np63 $\alpha$  promotes tumorigenesis via induction of *Lsh*-mediated bypass of OIS.

Another major finding of this work is that  $\Delta$ Np63 $\alpha$ 's oncogenic role is associated with its ability to maintain the K15-positive stem cell population. There is accumulating evidence that p63, and in particular the  $\Delta$ Np63 $\alpha$  isoform, maintains the proliferative capacity of epithelial stem cells of the skin, thymus, prostate, breast, and eye (Pellegrini et al., 2001; Senoo et al., 2007; Xin et al., 2007). However, the mechanisms by which p63 mediates stem-like proliferation has not been established. Our identification of *Lsh*, a chromatin-remodeling protein robustly expressed in human embryonic stem cells (Assou et al., 2007), as a  $\Delta$ Np63 $\alpha$  target suggests that p63 plays a role in epigenetic regulation of stemness. Our findings also raise interesting questions regarding the role of stem or stem-like cells in tumorigenesis. In some tumors, the cell of origin is suggested to be a normal somatic stem cell that has acquired mutations that allow it to transform (Barker et al., 2009). In other tumors, however, the cell of origin is suggested to be a more mature progenitor or committed daughter cell that acquired mutations that make it function more like a stem cell, thereby promoting tumorigenesis (Jamieson et al., 2004). Our data support the fact that  $\Delta$ Np63 $\alpha$  promotes aberrant proliferation of the K15 population of stem cells in the skin, providing evidence that these are the tumor-initiating population in SCC. In contrast with tumors such as pancreatic intraepithelial neoplasia and malignant astrocytomas, in which Nestin-positive stem/progenitor cells are the source of malignancy after oncogene activation or tumor-suppressor loss (Alcantara Llaguno et al., 2009; Carrière et al., 2007; Dai et al., 2001), our data suggest that  $\Delta$ Np63 $\alpha$  does not target the

Nestin population in this context. Our finding that  $\Delta Np63\alpha$  affects a multipotent stem cell population and identification of Lsh as a critical component of senescence bypass concomitant with the acquisition/maintenance of stem-like features and tumorigenesis suggests that epigenetic events play a critical role in this process.

## EXPERIMENTAL PROCEDURES

### Keratinocyte Culture, Retroviral Infection, and Hairpin Design

Primary keratinocyte cultures were prepared as previously described (Keyes et al., 2005). Differentiation was induced in media + 1.5 mM  $\text{CaCl}_2$  for 6–96 hr. For GFP-stem cell assays, primary keratinocytes from *K15-GFP* (Morris et al., 2004) and *Nestin-GFP* transgenic (Mignone et al., 2004, 2007) mice were prepared and infected as described. GFP quantitation in *Nestin-GFP* and *K15-GFP* models was performed with Guava EasyCyte (Guava Technologies) and LSRII (BD Biosciences) FACS analyzer and FloJo software, respectively. Human HNSCC cells were previously described (Rocco et al., 2006). Western blotting and qRT-PCR were performed as described (Keyes et al., 2005) with primers listed (Supplemental Experimental Procedures). SA- $\beta$ -gal activity was performed as described (Keyes et al., 2005). Mouse Genome 430A 2.0 microarrays (Affymetrix) were used. siRNA's targeting *Lsh* (CCGTCGGAAATTAGTAAAGAA) were designed with the Biopredsi (<http://www.biopredsi.org/design.html>) website, cloned into LMP vectors (Dickins et al., 2005), evaluated for efficiency in mouse embryonic fibroblasts (mefs), and assessed by western blotting 2 days after drug selection. Keratinocyte infections were performed essentially as described previously (Keyes et al., 2005). In brief, retrovirus was produced by transiently transfecting the ecotropic Phoenix packaging cell line (G. Nolan, Stanford University, Stanford, CA). Two days after transfection, viral supernatant was collected, filtered, and added to the keratinocyte cultures. Two infections of 2 hr each were performed. After 2 days, keratinocytes were drug selected according to the different selection markers.

### Three-Dimensional Tissue Culture

Three-dimensional tissue culture was performed as previously described (Aranda et al., 2006), with 2.5% collagen:matrigel (BD Biosciences) 1:1 mix as matrix.  $5 \times 10^3$  cells were seeded onto 8-well chamber slides (BD Falcon) and fresh media containing collagen:matrigel was added every 2 days. Morphology was assessed by phase microscopy. For immunofluorescence, cultures were fixed and stained as described (Aranda et al., 2006), with anti-K14 polyclonal antibody AF64 (Covance Research Products Inc.) and Alexa Fluor secondary antibodies (Molecular Probes).

### Tumor Study

Cells were injected subcutaneously into athymic nude mice as outlined in Table S1. Tumor development was monitored by whole-body GFP imaging. Histopathology was performed by H.V. Immunohistochemistry was performed as described (Keyes et al., 2005). Animals were housed and handled according to Barcelona Biomedical Research Park Animal Facility (PRBB), Cold Spring Harbor Institutional Animal Care and Use Committee (IACUC), and Association for Accreditation of Laboratory Animal Care International (AAALAC) policies.

### Chromatin Immunoprecipitation

RAN keratinocytes were fixed in 1% formaldehyde, 10 min, room temperature, crosslinking stopped in 0.125 M glycine/PBS, washed in cold PBS, centrifuged 2000 rpm, 2 min, and resuspended to  $\sim 3 \times 10^6$  cells/ml in cell lysis buffer (5 mM HEPES [pH 7.9], 85 mM KCl, 0.5% NP40) + protease inhibitors and incubated on ice, 15 min. Homogenates were centrifuged at 5000 rpm, 5 min, 4°C, pellets resuspended in nuclear lysis buffer (50 mM Tris [pH 8], 10 mM EDTA [pH 8], 1% SDS) + protease inhibitors and incubated on ice, 20 min. Nuclear lysates were sonicated with a Diagenode Sonicator and centrifuged at 12,000 rpm, 10 min, 4°C. Supernatants were diluted in IP buffer (150 mM NaCl, 20 mM Tris [pH 8], 2 mM EDTA) with Protein-A/G beads (previously pre-blocked with sheared salmon sperm DNA and BSA and precleared for >2 hr at 4°C). Preblocked protein A/G beads were incubated with 4A4 antibody (Santa Cruz, SC-8431) or  $\alpha$ -integrin 1 $\alpha$  antibody (Santa Cruz, SC-8978) in IP buffer

(2 hr, 4°C), centrifuged, washed with IP buffer, and incubated with precleared chromatin overnight at 4°C. Beads were pelleted, washed sequentially with low salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris [pH 8], 150 mM NaCl), high salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris [pH 8], 500 mM NaCl), LiCl buffer (0.25 M LiCl, 1% NP40, 1%  $\text{Na}^{+2}$  deoxycholate, 1 mM EDTA, 10 mM Tris [pH 8]), and TE buffer. Chromatin was eluted in elution buffer (100 mM  $\text{NaHCO}_3$ , 1% SDS) overnight at 65°C, collected by centrifugation, and incubated with proteinase K (30 min, 37°C). DNA was extracted and PCR amplified with specific primers (see Figure S5, Supplemental Experimental Procedures).

p63 consensus-binding sites *WDDCNDGHHH* {1–25} *WDDCNDGHHH* were identified with modifications of previously published motifs (Ort and Sinha, 2006; Yang et al., 2006). The *Lsh* promoter was identified with the TRED database (<http://rulai.cshl.edu/cgi-bin/TRED/tred.cgi?process=home>) and p63 consensus sites were identified  $\sim 1300$  bp upstream of the TSS (38284774 of NM\_008234) and  $\sim 2500$  bp upstream of the TSS (47110038 of NT\_030059) of mouse and human *LSH*, respectively (see Figure S5).

## SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and one table and can be found with this article online at doi:10.1016/j.stem.2010.12.009.

## ACKNOWLEDGMENTS

We thank Johannes Zuber, Michael Hemann, and Scott Lowe for assistance and advice; Yuri Lazebnik and Dominik Duelli for DMBA-treated tissues; Chris Johns and Sohail Khan for microarray analysis; and Leif Ellisen and James W. Rocco for HNSCC cell lines. W.M.K. was funded by the Spanish Ministry of Science and Innovation (Plan Nacional –SAF2010-18829). A.A.M. was funded by an ACS Research Scholar Award (RSG-06-190-01-MGO).

Received: July 3, 2009

Revised: October 21, 2010

Accepted: November 15, 2010

Published: February 3, 2011

## REFERENCES

- Alcantara Llaguno, S., Chen, J., Kwon, C.H., Jackson, E.L., Li, Y., Burns, D.K., Alvarez-Buylla, A., and Parada, L.F. (2009). Malignant astrocytomas originate from neural stem/progenitor cells in a somatic tumor suppressor mouse model. *Cancer Cell* 15, 45–56.
- Aranda, V., Haire, T., Nolan, M.E., Calarco, J.P., Rosenberg, A.Z., Fawcett, J.P., Pawson, T., and Muthuswamy, S.K. (2006). Par6-aPKC uncouples ErbB2 induced disruption of polarized epithelial organization from proliferation control. *Nat. Cell Biol.* 8, 1235–1245.
- Assou, S., Le Carrou, T., Tondeur, S., Ström, S., Gabelle, A., Marty, S., Nadal, L., Pantescio, V., Réme, T., Hugnot, J.P., et al. (2007). A meta-analysis of human embryonic stem cells transcriptome integrated into a web-based expression atlas. *Stem Cells* 25, 961–973.
- Barker, N., Ridgway, R.A., van Es, J.H., van de Wetering, M., Begthel, H., van den Born, M., Danenberg, E., Clarke, A.R., Sansom, O.J., and Clevers, H. (2009). Crypt stem cells as the cells-of-origin of intestinal cancer. *Nature* 457, 608–611.
- Blanpain, C., Lowry, W.E., Geoghegan, A., Polak, L., and Fuchs, E. (2004). Self-renewal, multipotency, and the existence of two cell populations within an epithelial stem cell niche. *Cell* 118, 635–648.
- Brunner, H.G., Hamel, B.C., and Van Bokhoven, H. (2002). The p63 gene in EEC and other syndromes. *J. Med. Genet.* 39, 377–381.
- Cao, L., Li, W., Kim, S., Brodie, S.G., and Deng, C.X. (2003). Senescence, aging, and malignant transformation mediated by p53 in mice lacking the Brca1 full-length isoform. *Genes Dev.* 17, 201–213.



- Carrière, C., Seeley, E.S., Goetze, T., Longnecker, D.S., and Korc, M. (2007). The Nestin progenitor lineage is the compartment of origin for pancreatic intra-epithelial neoplasia. *Proc. Natl. Acad. Sci. USA* 104, 4437–4442.
- Celli, J., Duijff, P., Hamel, B.C., Bamshad, M., Kramer, B., Smits, A.P., Newbury-Ecob, R., Hennekam, R.C., Van Buggenhout, G., van Haeringen, A., et al. (1999). Heterozygous germline mutations in the p53 homolog p63 are the cause of EEC syndrome. *Cell* 99, 143–153.
- Collado, M., Gil, J., Efeyan, A., Guerra, C., Schuhmacher, A.J., Barradas, M., Benguría, A., Zaballos, A., Flores, J.M., Barbacid, M., et al. (2005). Tumour biology: Senescence in premalignant tumours. *Nature* 436, 642.
- Dai, C., Celestino, J.C., Okada, Y., Louis, D.N., Fuller, G.N., and Holland, E.C. (2001). PDGF autocrine stimulation dedifferentiates cultured astrocytes and induces oligodendrogliomas and oligoastrocytomas from neural progenitors and astrocytes in vivo. *Genes Dev.* 15, 1913–1925.
- Dennis, K., Fan, T., Geiman, T., Yan, Q., and Muegge, K. (2001). Lsh, a member of the SNF2 family, is required for genome-wide methylation. *Genes Dev.* 15, 2940–2944.
- Di Como, C.J., Urist, M.J., Babayan, I., Drobnjak, M., Hedvat, C.V., Teruya-Feldstein, J., Pohar, K., Hoos, A., and Cordon-Cardo, C. (2002). p63 expression profiles in human normal and tumor tissues. *Clin. Cancer Res.* 8, 494–501.
- Dickins, R.A., Hemann, M.T., Zilfou, J.T., Simpson, D.R., Ibarra, I., Hannon, G.J., and Lowe, S.W. (2005). Probing tumor phenotypes using stable and regulated synthetic microRNA precursors. *Nat. Genet.* 37, 1289–1295.
- Donehower, L.A., Harvey, M., Slagle, B.L., McArthur, M.J., Montgomery, C.A., Jr., Butel, J.S., and Bradley, A. (1992). Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. *Nature* 356, 215–221.
- Dotto, G.P. (1999). Signal transduction pathways controlling the switch between keratinocyte growth and differentiation. *Crit. Rev. Oral Biol. Med.* 10, 442–457.
- Fernandes, K.J., Toma, J.G., and Miller, F.D. (2008). Multipotent skin-derived precursors: adult neural crest-related precursors with therapeutic potential. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 363, 185–198.
- Flores, E.R., Sengupta, S., Miller, J.B., Newman, J.J., Bronson, R., Crowley, D., Yang, A., McKeon, F., and Jacks, T. (2005). Tumor predisposition in mice mutant for p63 and p73: evidence for broader tumor suppressor functions for the p53 family. *Cancer Cell* 7, 363–373.
- Geiman, T.M., Tessarollo, L., Anver, M.R., Kopp, J.B., Ward, J.M., and Muegge, K. (2001). Lsh, a SNF2 family member, is required for normal murine development. *Biochim. Biophys. Acta* 1526, 211–220.
- Gemenetzidis, E., Bose, A., Riaz, A.M., Chaplin, T., Young, B.D., Ali, M., Sugden, D., Thurlow, J.K., Cheong, S.C., Teo, S.H., et al. (2009). FOXM1 up-regulation is an early event in human squamous cell carcinoma and it is enhanced by nicotine during malignant transformation. *PLoS ONE* 4, e4849.
- Guo, X., Keyes, W.M., Papazoglu, C., Zuber, J., Li, W., Lowe, S.W., Vogel, H., and Mills, A.A. (2009). TAp63 induces senescence and suppresses tumorigenesis in vivo. *Nat. Cell Biol.* 11, 1451–1457.
- Hibi, K., Trink, B., Patturajan, M., Westra, W.H., Caballero, O.L., Hill, D.E., Ratovitski, E.A., Jen, J., and Sidransky, D. (2000). AIS is an oncogene amplified in squamous cell carcinoma. *Proc. Natl. Acad. Sci. USA* 97, 5462–5467.
- Huang, J., Fan, T., Yan, Q., Zhu, H., Fox, S., Issaq, H.J., Best, L., Gangi, L., Munroe, D., and Muegge, K. (2004). Lsh, an epigenetic guardian of repetitive elements. *Nucleic Acids Res.* 32, 5019–5028.
- Jacks, T., Remington, L., Williams, B.O., Schmitt, E.M., Halachmi, S., Bronson, R.T., and Weinberg, R.A. (1994). Tumor spectrum analysis in p53-mutant mice. *Curr. Biol.* 4, 1–17.
- Jamieson, C.H., Ailles, L.E., Dylla, S.J., Muijtjens, M., Jones, C., Zehnder, J.L., Gotlib, J., Li, K., Manz, M.G., Keating, A., et al. (2004). Granulocyte-macrophage progenitors as candidate leukemic stem cells in blast-crisis CML. *N. Engl. J. Med.* 351, 657–667.
- Keyes, W.M., Wu, Y., Vogel, H., Guo, X., Lowe, S.W., and Mills, A.A. (2005). p63 deficiency activates a program of cellular senescence and leads to accelerated aging. *Genes Dev.* 19, 1986–1999.
- Keyes, W.M., Vogel, H., Koster, M.I., Guo, X., Qi, Y., Petherbridge, K.M., Roop, D.R., Bradley, A., and Mills, A.A. (2006). p63 heterozygous mutant mice are not prone to spontaneous or chemically induced tumors. *Proc. Natl. Acad. Sci. USA* 103, 8435–8440.
- Lawson, D.A., Xin, L., Lukacs, R.U., Cheng, D., and Witte, O.N. (2007). Isolation and functional characterization of murine prostate stem cells. *Proc. Natl. Acad. Sci. USA* 104, 181–186.
- Mignone, J.L., Kukekov, V., Chiang, A.S., Steindler, D., and Enikolopov, G. (2004). Neural stem and progenitor cells in nestin-GFP transgenic mice. *J. Comp. Neurol.* 469, 311–324.
- Mignone, J.L., Roig-Lopez, J.L., Fedtsova, N., Schones, D.E., Manganas, L.N., Maletic-Savatic, M., Keyes, W.M., Mills, A.A., Gleiberman, A., Zhang, M.Q., and Enikolopov, G. (2007). Neural potential of a stem cell population in the hair follicle. *Cell Cycle* 6, 2161–2170.
- Mills, A.A., Zheng, B., Wang, X.J., Vogel, H., Roop, D.R., and Bradley, A. (1999). p63 is a p53 homologue required for limb and epidermal morphogenesis. *Nature* 398, 708–713.
- Mills, A.A., Qi, Y., and Bradley, A. (2002). Conditional inactivation of p63 by Cre-mediated excision. *Genesis* 32, 138–141.
- Missero, C., Di Cunto, F., Kiyokawa, H., Koff, A., and Dotto, G.P. (1996). The absence of p21Cip1/WAF alters keratinocyte growth and differentiation and promotes ras-tumor progression. *Genes Dev.* 10, 3065–3075.
- Morris, R.J., Liu, Y., Marles, L., Yang, Z., Trempus, C., Li, S., Lin, J.S., Sawicki, J.A., and Cotsarelis, G. (2004). Capturing and profiling adult hair follicle stem cells. *Nat. Biotechnol.* 22, 411–417.
- Muegge, K. (2005). Lsh, a guardian of heterochromatin at repeat elements. *Biochem. Cell Biol.* 83, 548–554.
- Myant, K., and Stancheva, I. (2008). LSH cooperates with DNA methyltransferases to repress transcription. *Mol. Cell. Biol.* 28, 215–226.
- Narita, M., and Lowe, S.W. (2005). Senescence comes of age. *Nat. Med.* 11, 920–922.
- Ortt, K., and Sinha, S. (2006). Derivation of the consensus DNA-binding sequence for p63 reveals unique requirements that are distinct from p53. *FEBS Lett.* 580, 4544–4550.
- Pellegrini, G., Dellambra, E., Golisano, O., Martinelli, E., Fantozzi, I., Bondanza, S., Ponzin, D., McKeon, F., and De Luca, M. (2001). p63 identifies keratinocyte stem cells. *Proc. Natl. Acad. Sci. USA* 98, 3156–3161.
- Rocco, J.W., Leong, C.O., Kuperwasser, N., DeYoung, M.P., and Ellisen, L.W. (2006). p63 mediates survival in squamous cell carcinoma by suppression of p73-dependent apoptosis. *Cancer Cell* 9, 45–56.
- Schaetzlein, S., Kodandamireddy, N.R., Ju, Z., Lechel, A., Stepczynska, A., Lill, D.R., Clark, A.B., Rudolph, C., Kuhnel, F., Wei, K., et al. (2007). Exonuclease-1 deletion impairs DNA damage signaling and prolongs lifespan of telomere-dysfunctional mice. *Cell* 130, 863–877.
- Senoo, M., Pinto, F., Crum, C.P., and McKeon, F. (2007). p63 is essential for the proliferative potential of stem cells in stratified epithelia. *Cell* 129, 523–536.
- Serrano, M., Lin, A.W., McCurrach, M.E., Beach, D., and Lowe, S.W. (1997). Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. *Cell* 88, 593–602.
- Su, X., Paris, M., Gi, Y.J., Tsai, K.Y., Cho, M.S., Lin, Y.L., Biernaskie, J.A., Sinha, S., Prives, C., Pevny, L.H., et al. (2009). TAp63 prevents premature aging by promoting adult stem cell maintenance. *Cell Stem Cell* 5, 64–75.
- Sun, L.Q., Lee, D.W., Zhang, Q., Xiao, W., Raabe, E.H., Meeker, A., Miao, D., Huso, D.L., and Arceci, R.J. (2004). Growth retardation and premature aging phenotypes in mice with disruption of the SNF2-like gene, PASG. *Genes Dev.* 18, 1035–1046.
- Toma, J.G., McKenzie, I.A., Bagli, D., and Miller, F.D. (2005). Isolation and characterization of multipotent skin-derived precursors from human skin. *Stem Cells* 23, 727–737.
- Tonon, G., Brennan, C., Protopopov, A., Maulik, G., Feng, B., Zhang, Y., Khatri, D.B., You, M.J., Aguirre, A.J., Martin, E.S., et al. (2005). Common and contrasting genomic profiles among the major human lung cancer subtypes. *Cold Spring Harb. Symp. Quant. Biol.* 70, 11–24.

- Tumbar, T., Guasch, G., Greco, V., Blanpain, C., Lowry, W.E., Rendl, M., and Fuchs, E. (2004). Defining the epithelial stem cell niche in skin. *Science* 303, 359–363.
- Xi, S., Zhu, H., Xu, H., Schmidtman, A., Geiman, T.M., and Muegge, K. (2007). Lsh controls Hox gene silencing during development. *Proc. Natl. Acad. Sci. USA* 104, 14366–14371.
- Xin, L., Lukacs, R.U., Lawson, D.A., Cheng, D., and Witte, O.N. (2007). Self-renewal and multilineage differentiation in vitro from murine prostate stem cells. *Stem Cells* 25, 2760–2769.
- Yang, A., Kaghad, M., Wang, Y., Gillett, E., Fleming, M.D., Dötsch, V., Andrews, N.C., Caput, D., and McKeon, F. (1998). p63, a p53 homolog at 3q27-29, encodes multiple products with transactivating, death-inducing, and dominant-negative activities. *Mol. Cell* 2, 305–316.
- Yang, A., Schweitzer, R., Sun, D., Kaghad, M., Walker, N., Bronson, R.T., Tabin, C., Sharpe, A., Caput, D., Crum, C., and McKeon, F. (1999). p63 is essential for regenerative proliferation in limb, craniofacial and epithelial development. *Nature* 398, 714–718.
- Yang, A., Zhu, Z., Kapranov, P., McKeon, F., Church, G.M., Gingeras, T.R., and Struhl, K. (2006). Relationships between p63 binding, DNA sequence, transcription activity, and biological function in human cells. *Mol. Cell* 24, 593–602.